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Characterisation of high-altitude *Artemia* populations from the Qinghai-Tibet Plateau, PR China

Gilbert Van Stappen[1], Liying Sui[2], Naihong Xin[2] & Patrick Sorgeloos[1]
[1]*Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Rozier 44, B-9000 Gent, Belgium*
Tel: 32-9-2643754. Fax: 32-9-2644193. E-mail: gilbert.vanstappen@UGent.be
[2]*Salt Research Institute, Yingkou Road 831, 300450 Tanggu, Tianjin, PR China*

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Abstract

The brine shrimp *Artemia* was discovered in a number of saline lakes on the Qinghai-Tibet Plateau, widely diverging in chemical composition. Several lakes were athalassohaline, with relatively high amounts of trace elements. Common environmental factors are their high altitude (exceeding 4500 m) and the low average annual temperatures. A number of *Artemia* populations in this area were analysed to assess their preference for low temperatures and an athalassohaline medium. Furthermore, their characteristics were compared with *Artemia tibetiana*, the species recently described for one lake in this area. All samples contained a variable mixture of parthenogenetic and bisexual individuals. A cross-breeding test of the sample from Jingyu Lake showed cross-fertility both with *A. tibetiana* and *A. sinica.* All populations showed similarities to *A. tibetiana*: a large cyst diameter and naupliar length, high HUFA content and a high tolerance to low temperatures, as compared to the control *A. franciscana* samples. These can thus be considered as recurrent characteristics of the populations from the high-altitude low-temperature environment on the Qinghai-Tibet Plateau, although further research is needed to identify their exact species status.

Introduction

The Qinghai-Tibet Plateau, located in southwest China, has an average elevation exceeding 4500 m. About 350 saline lakes are situated in this area and have a wide diversity of geological background and chemical composition (Zheng et al., 1993).

About 16 *Artemia* sites on the Qinghai-Tibet Plateau are reported by Xin et al. (1994) and Zheng (1997). New habitats are being explored and new cyst material is being collected. However, knowledge about these particular *Artemia* biotopes advances at a rather slow pace, due to the difficult accessibility of the area and the logistic problems for cyst harvesters. Consequently only limited amounts of samples have been collected and analysed. Unfortunately their exact origin and background are not always easy to trace, which may hinder the interpretation of analytical results. Finally, inconsistent transcription and random use of local Tibetan and/or Chinese toponyms add to the confusion.

As compared with the lakes elsewhere in China, these saline lakes show higher contents of trace elements such as B, Li, Cs, Rb and As (Zheng, 1997), to the extent that commercial extraction of lithium and boron is a local industrial activity (e.g. in Lake Zabuye) (Zheng, 2002). This environment is further characterized by its high altitude (as high as 4900 m), and low temperatures (average annual air temperature between –5 and +1 °C; Zheng, 1997).

Insofar as Chinese scientific research is accessible to the international community, most studies focused on the *Artemia* population from Lagkor Co (Liu et al., 1998ab). Through a multidisciplinary approach

Abatzopoulos et al. (1998, 2002) identified this population as a new bisexual species, *Artemia tibetiana*. Sun et al. (1999), using Amplified Fragment Length Polymorphism (AFLP) of different Chinese *Artemia* species and strains, succeeded in differentiating *A. tibetiana* from *A. sinica*, the other bisexual species from continental China. Han et al. (1999) illustrated the different fatty acid metabolism of this species after enrichment, in comparison with *A. franciscana*. Clegg et al. (2001) reported on its high sensitivity for heat stress, probably an adaptation to its cold natural environment.

Field data of the brine shrimp population of Lagkor Co or any other population on the Plateau are extremely scarce and are generally limited to momentary observations (Zheng, 1997). The species status of the populations in other lakes has also not been confirmed. It is not clear to what extent the characteristics, as previously described for *A. tibetiana*, also occur in other *Artemia* populations from the same area. This study aims to contribute to the knowledge of the anostracan biodiversity in this area, by analysing the characteristics of a number of *Artemia* samples from the Qinghai-Tibet Plateau that have recently become available.

Materials and methods

Cyst samples

Seven samples originating from the Qinghai-Tibet plateau were studied (Table 1). The number of analyses run on each sample depended on the available cyst quantity and their hatching percentage. The exact geographical background of one sample ('Tibet A') was unknown.

Biometrical characteristics

The cyst diameter, chorion thickness and nauplius length were determined (n = 100), according to the methodology as described in Vanhaecke & Sorgeloos (1980).

Nutritional content: level of highly unsaturated fatty acids (HUFA's)

The fatty acid composition of the *Artemia* nauplii was analysed by a direct transmethylation method according to a modified procedure of Lepage & Roy (1984). The resulting fatty acid methyl esters (FAME) were separated and identified on a Chrompack CP 9001 gas chromatograph equipped with autosampler and a temperature programmable on-column injector (TPOCI). Identification was based on standard reference mixtures (Nu-Chek-Prep, U.S.A.); integration and calculations were done with the Maestro, Chrompack, software program.

Impact of environmental conditions

A number of laboratory tests were performed to assess the adaptation of the strains to the conditions as prevailing in their natural habitat: temperature, salinity and ionic composition of the medium.

a) hatching at different temperatures

The samples of Tibet A, Bong Co, Jingyu Lake, Haiyan Lake and Co Qen were hatched in triplicate at standard hatching conditions (35 $g.l^{-1}$, Instant Ocean® synthetic sea salt mixture, continuous illumination of 2000 lux provided by TL lamps; Lavens & Sorgeloos, 1996), but at different temperatures: 10, 16, 21 and 28 ± 0.5 °C. These temperatures were obtained by installing the 800 ml glass cylindroconical hatching recipients in an air-conditioned room (10 and 16 °C) or in a heated water bath (21 and 28 °C). The hatching process was followed over a total incubation period of 144 h, with measurements taken every 24 h. *Artemia franciscana* cysts (San Francisco Bay, SFB, California, USA; ARC code 1258) were used as control.

b) hatching in medium of different salinity, ionic composition and temperature

Tibet A and Lagkor Co cysts were hatched in the thalassohaline Dietrich & Kalle artificial seawater (Parsons et al., 1984) and in an artificially made Lagkor Co water (Table 2) using a simplified formula, based on ionic data as provided by Zheng (1997) (detailed information about the ionic composition of the other lakes was not available). The hatching was assessed at salinities of 15, 35 and 80 $g.l^{-1}$ for each medium. This comparative test was run both at 21 and 28 ± 0.5 °C (by use of a heated water bath). All other conditions were standard (Lavens & Sorgeloos, 1996) and identical to the previous test. The test was run in triplicate for each combination of variables. Hatching was followed over a total incubation period of 48 h, with measurements taken every 24 h. *A. franciscana* cysts (Great Salt Lake, GSL, Utah, U.S.A.; commercial batch) were used as control, as the San Francisco

Table 1. Cyst samples: origin, ARC cyst code, geographical and hydrochemical parameters (Zheng, 1997, and data provided by Salt Research Institute, Tanggu, China); H% = hatching percentage of raw sample in standard conditions (Lavens & Sorgeloos, 1996) upon arrival at ARC

	ARC code	Elevation (m)	Surface area (km^2)	Longitude (E)	Latitude (N)	Ionic composition	H%
Tibet A (unknown origin)	1346	–	–	–	–	–	62.4
Lagkor Co	1348	4490	92	84°13′	32° 03′	Carbonate	11.8
Bong Co	1462	4664	140	91° 09′	31° 13′	–	6.7
Bozi Co	1461	4663	25	86° 07′	30° 28′	–	21.7
Jingyu Lake	1524	4720	300	89° 09′	36° 03′	$MgSO_4$	65.6
Haiyan Lake	1525	–	–	100° 11′	36° 03′	–	54.8
Co Qen	1526	–	–	85° 09′	30° 59′	Na_2SO_4	36.8

Bay sample (ARC code 1258) had shown low hatching in the previous test.

c) survival in medium of different salinity, ionic composition and temperature

Tibet A cysts were hatched at standard conditions (35 $g.l^{-1}$ Instant Ocean® artificial seawater, 28 °C; Lavens & Sorgeloos, 1996). Two hundred instar I nauplii were transferred into glass cylindroconical recipients with 800 ml culture medium, and grown for a total period of 17 days at the same combinations of temperature, salinity and ionic composition as described under b (the lowest salinity, 15 $g.l^{-1}$, was not included in this test). As the instar I nauplius is resistant to osmotic shocks (Sorgeloos, 1980), no acclimation period was provided. Bottom aeration was provided continuously and a 12/12 photoperiod (light intensity 2000 lux) was maintained. Each treatment was run in triplicate. The test animals were fed a standard diet, based on the unicellular alga *Dunaliella tertiolecta* Butch (adapted to the respective salinities) and the yeast-based Lanzy PZ® (INVE N.V., Belgium) (Coutteau et al., 1992; Nguyen Thi, 2000). Survival was determined (and water renewed) at day 4, 8, 11, 14 and 17.

d) heat shock test

Cysts from Jingyu Lake and Co Qen were subjected to a heat shock test, as described by Clegg et al. (2001). Hydrated cysts were gradually heated from 22.0 to 50.0 °C. After being maintained for 15, 30, 60 and 80 min at 50.0 °C, the cysts were incubated again at 22.0 °C and the hatching was assessed. Cysts from Lagkor Co, San Francisco Bay (ARC code 1364) and Vietnam (Vinh Chau, ARC code 1349) were included as reference strains, in order to compare with available literature data.

Table 2. Formulation of Lagkor Co and Dietrich & Kalle artificial media; salt contents of stock solution ($g.l^{-1}$ medium; to be diluted to experimental salinities)

Salt	Lagkor Co	Dietrich & Kalle
NaCl	11.64	67.90
$MgCl_2 \bullet 6H_2O$	11.34	30.77
$CaCl_2$	0.43	3.27
KCl	8.56	1.94
$Na_2SO_4 \bullet 10\ H_2O$	111.40	25.74
$Na\ HCO_3$	6.84	1.14
H_3BO_3	4.19	0.008
Na_2CO_3	3.66	–

Species characterization of strains

a) sex ratio

In order to assess the type of reproduction (parthenogenetic or bisexual), cysts of all populations (except Lagkor Co, which had been defined as *A. tibetiana*) were hatched in standard conditions (Lavens & Sorgeloos, 1996). Instar I nauplii of each sample were subsequently cultured in 800 ml glass cylindroconical recipients. The test was performed at 21 ± 1 °C in 80 $g.l^{-1}$ Instant Ocean® artificial seawater, 2000 lux illumination with photoperiod 12/12, with a diet of exclusively *Dunaliella tertiolecta* (Coutteau et al., 1992). From the age of seven days onwards, 100–120

animals of each sample were raised individually in 50 ml Falcon tubes for a period of 1 month (or until all animals had died) in the same culture conditions. Water was renewed twice a week. When sexual maturity was attained, the number of males and females was determined, as well as the number of females releasing nauplii and/or cysts and ovigerous females that didn't spawn. In case of cyst release, the viability of the offspring was assessed by hatching the cysts in standard conditions (Lavens & Sorgeloos, 1996) after two weeks storage in brine at –18 °C to break diapause.

b) cross-breeding

In view of the mixed status of the samples (as revealed by the sex ratio test), a cross breeding test was only performed with the Jingyu Lake sample, which had the lowest fraction of parthenogenetic females. Reciprocal crosses of the Jingyu strain were performed with *A. tibetiana* (Lagkor Co, ARC code 1348), *A. sinica* (Yuncheng, China, ARC code 1218) and *A. franciscana* (San Francisco Bay, USA, ARC code 1364) (see Table 7 for design of crosses).

Cysts were hatched in standard conditions, and nauplii were individually raised in 50 ml Falcon tubes (21 ± 1 °C, 80 $g.l^{-1}$ Instant Ocean® water, 2000 lux illumination, photoperiod 12/12, standard diet of *Dunaliella tertiolecta*). As soon as sexual differentiation occurred, males and females were paired (n = 10) and couples were raised separately in 50 ml Falcon tubes in the same culture conditions. Twice per week water was renewed and F1 offspring (cysts or nauplii) was counted. F1 cysts were stored at –18 °C for a minimum period of 2 weeks, while dehydrated in 300 $g.l^{-1}$ brine, to break the state of diapause. After an acclimation period of one week at room temperature, F1 cysts were hatched in standard conditions, and if sufficient animals were available, animals were paired (n=10) following the same procedure as for the parental generation. Ovoviviparously generated F1 nauplii were directly raised in the same conditions, and used for further crossing, if available in sufficient numbers. As the offspring of each set of 10 replicates was pooled, cross-fertility was assessed by the hatching percentage of the cysts produced, the encystment rate, and the number of ovoviviparously reproducing females in each combination.

Reciprocal crosses were continued until the F3 generation for those combinations where sufficient animals were produced. Each generation was cultured, and the offspring (cysts or nauplii) counted, for a maximum period of 30 days (or until death of all individuals). Dead individuals were replaced from the stock of individually cultured animals for the first two weeks; if males died after this period, the surviving female was further monitored.

Table 3. Biometric data of cyst samples (n=100). Values within the same column, sharing the same superscript, are not significantly different (one-way ANOVA at $p = 0.05$)

Sample	Cyst diameter (μm)	Chorion thickness (μm)	Instar I naupliar length (μm)
Tibet A	306.3 ± 20.3^{bc}	5.7	590.5 ± 36.1^{b}
Bong Co	295.7 ± 14.8^{d}	3.6	Not analysed
Bozi Co	284.5 ± 16.4^{e}	3.8	Not analysed
Jingyu Lake	320.0 ± 13.7^{ab}	13.3	607.1 ± 34.6^{a}
Haiyan Lake	291.2 ± 14.3^{d}	13.3	540.2 ± 31.6^{d}
Co Qen	312.1 ± 19.6^{abc}	11.2	558.3 ± 35.5^{c}

Statistical processing

Differences in cyst diameter and instar I naupliar length between strains were analysed by one-way ANOVA.

For the Great Salt Lake, Lagkor Co and Tibet A samples, the effects of ionic composition, temperature and salinity on hatching after 24 h hatching incubation were tested by a three-way ANOVA. The same analysis was performed with the 48 h hatching incubation data.

For the Tibet A sample, the effects of ionic composition, temperature and salinity on survival after 4, 8, 11, 14 and 17 days of culture were tested by three-way ANOVA's.

All data were tested for normality and homogeneity of variance before ANOVA was done. Tukey's Honest Significant Difference (HSD) test was performed to identify differences among means and significance was accepted at $p<0.05$.

Results

Biometrical characteristics (Table 3)

Average cyst diameters ranged between 284.5 μm (Bozi Co) and 320.0 μm (Jingyu Lake). Significant differences (one-way ANOVA, $p < 0.05$) in cyst diameter were found between the samples, with the

Table 4. HUFA analysis of cyst samples

FAME ($mg.g^{-1}$ dry weight)	Lagkor Co	Bong Co	Bozi Co	Jingyu L.	Haiyan L.	Co Qen
18:2(ω-6)t	0.8	0.6	0.6	0.5	0.5	0.5
18:2(ω-6)c	5.4	5.7	4.5	5.9	5.7	6.6
18:3(ω-3)	7.5	0.2	0.5	4.9	12.1	4.4
20:4(ω-6)	7.4	2.1	3.1	4.4	1.8	2.5
20:5(ω-3)	43.0	29.4	21.6	42.7	31.7	30.7
22:6(ω-3)	0.7	0.5	0.6	0.9	0.3	1.1
$\Sigma(\omega\text{-}3) \geq 20{:}3(\omega\text{-}3)$	45.0	31.2	23.2	45.0	33.8	33.1
$\Sigma(\omega\text{-}6) \geq 18{:}2(\omega\text{-}6)$t	15.5	8.8	9.0	11.2	8.3	9.9

Jingyu Lake, Co Qen and Tibet A samples being significantly bigger, and the Bozi Co sample significantly smaller than the others.

Average instar I naupliar length ranged between 540.2 μm (Haiyan Lake) and 607.1 μm (Jingyu Lake). All values (4 samples analysed) were significantly different from one another (one-way ANOVA, $p < 0.05$). Both low (3.6 μm in Bozi Co) and high (13.3 μm for Jingyu and Haiyan Lakes) values were recorded for the chorion thickness.

Nutritional content: level of highly unsaturated fatty acids (HUFA's) (Table 4)

Cyst samples showed total ω-3 HUFA levels ($\geq$20:3(ω-3)) ranging between 23.2 (Bozi Co) and 45.0 $mg.g^{-1}$ dry weight (Lagkor Co and Jingyu Lake). Levels of 20:5(ω-3) (eicosapentaenoic acid, EPA) ranged between 21.6 and 43.0 $mg.g^{-1}$ dry weight for Bozi Co and Lagkor Co cysts, respectively. The Co Qen sample showed the highest value of 22:6(ω-3) (docosahexaenoic acid, DHA): 1.1 $mg.g^{-1}$. The values for other HUFA's with aquaculture relevance, e.g. linoleic acid 18:2(ω-6), linolenic acid 18:3(ω-3) and arachidonic acid 20:4(ω-6), were variable.

Impact of environmental conditions

a) hatching at different temperatures (Fig. 1)

Samples showed very different hatching levels (see Table 1) under standard conditions. To facilitate comparison values for each sample were plotted as a fraction (%) of the reference value obtained at the standard temperature 28 °C after 48 hr. At 10 °C, Tibet A, Jingyu, Haiyan and Co Qen had started hatching after 72 h incubation period. The first hatching for Bong Co at this temperature was observed at 96 h, whereas the first hatching for SFB only occurred at 120 h. Except for Co Qen and SFB, the final hatching obtained in the range 10–21 °C was higher or similar than at the reference temperature of 28 °C. Generally, for all samples analysed, the hatching rate was delayed at lower temperatures.

b) hatching in medium of different salinity, ionic composition and temperature (Table 5)

After 24 h hatching incubation, both temperature and salinity had a significant effect on hatching (three-way ANOVA, $p < 0.05$) of all samples, whereas the type of medium only had a significant effect for the GSL strain. For all strains there was a significant interaction after 24 h incubation between hatching salinity on one hand, and ionic composition and temperature on the other. There was no significant interaction between ionic composition and salinity.

After 48 h hatching incubation, there was a significant effect ($p < 0.05$) of all factors and all interactions for all samples, with the exception of a non-significant temperature effect ($p > 0.05$) for GSL, and a non-significant interaction temperature vs. ionic composition for Tibet A.

The Great Salt Lake sample performed significantly better in the thalassohaline Dietrich & Kalle artificial medium, while both Tibet samples had higher hatching in the artificial Lagkor Co medium. In Dietrich & Kalle medium of 80 $g.l^{-1}$, both Tibet samples did not show any hatching at all within 48 h at both 21 and 28 °C. At lower salinities differences between both types of medium within each sample were non-significant ($p > 0.05$).

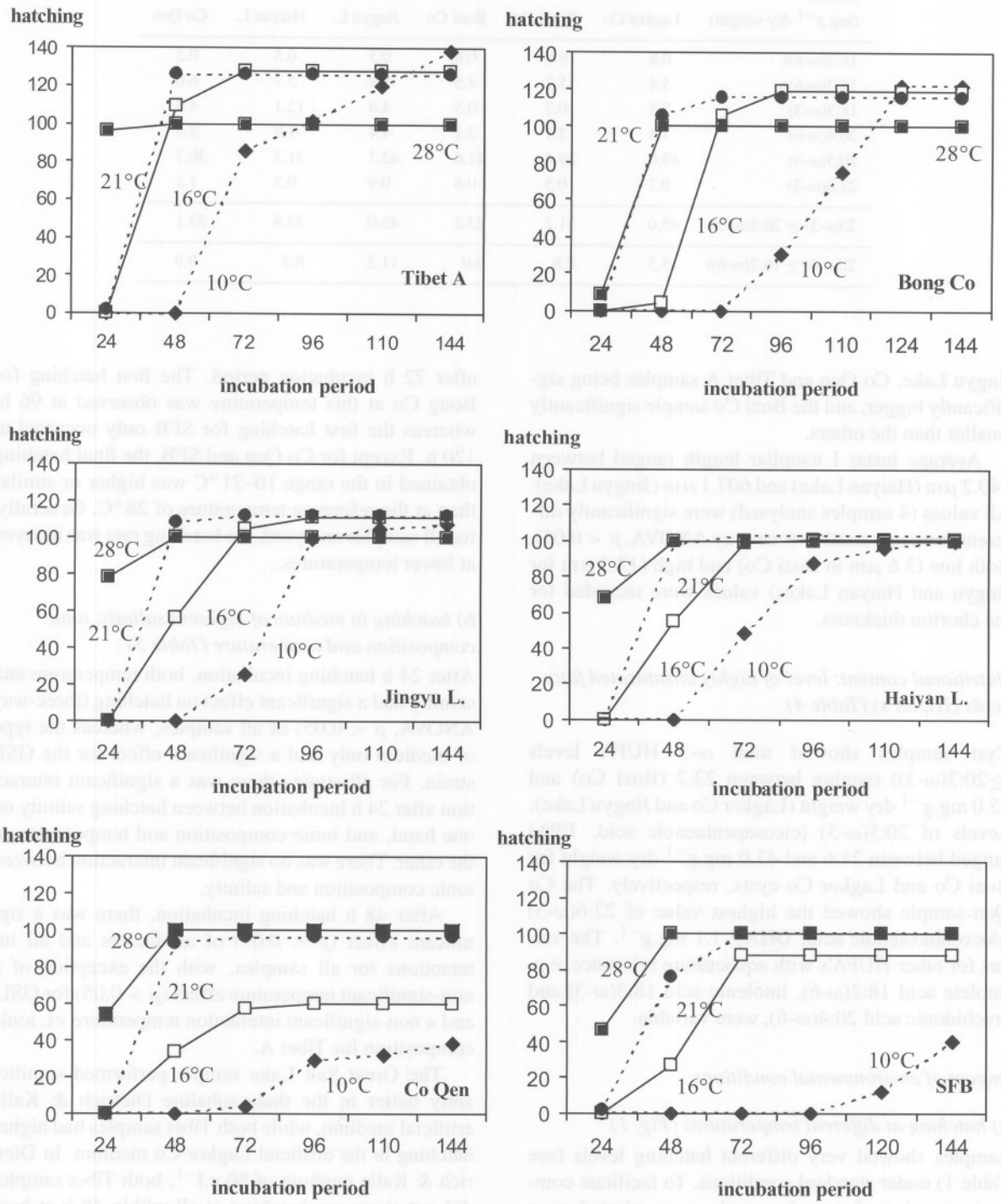

Figure 1. Hatching of cyst samples at different temperatures. Values are normalized to the value obtained at 28 °C after 48 hr (set as '100').

Table 5. Hatching in medium of different salinity, ionic composition and temperature. For each sample and incubation time (24 and 48 h) values with the same superscript are not significantly different (three-way ANOVA at $p = 0.05$); LC = Lagkor Co; DK = Dietrich & Kalle

Sample	Incubation time (hr)	Incubation temperature (°C)	15 g.l^{-1}		35 g.l^{-1}		80 g.l^{-1}	
			LC water	DK water	LC water	DK water	LC water	DK water
Tibet A	24	21	42.3 ± 12.8^{cd}	51.1 ± 1.7^{bcd}	22.6 ± 2.8^{e}	19.1 ± 3.4^{e}	0^{f}	0^{f}
		28	73.9 ± 0.8^{ab}	71.8 ± 2.8^{ab}	72.3 ± 2.1^{ab}	59.5 ± 11.0^{abc}	0^{f}	0^{f}
	48	21	72.7 ± 2.1^{abc}	78.7 ± 2.0^{ab}	74.8 ± 1.4^{abc}	76.3 ± 1.3^{ab}	68.0 ± 2.0^{bc}	0^{e}
		28	78.6 ± 4.8^{ab}	72.7 ± 3.0^{abc}	77.3 ± 2.1^{ab}	68.2 ± 2.6^{bc}	36.2 ± 5.0^{d}	0^{e}
Lagkor Co	24	21	5.0 ± 3.3^{def}	15.7 ± 6.0^{bcd}	12.5 ± 8.0^{bcde}	0^{ef}	0^{ef}	0^{ef}
		28	24.7 ± 1.4^{ab}	21.0 ± 2.9^{abcd}	0^{ef}	0^{ef}	0^{ef}	0^{ef}
	48	21	63.9 ± 7.9^{bcd}	63.8 ± 6.4^{bcd}	66.5 ± 6.1^{abcd}	58.9 ± 4.2^{bcd}	0^{f}	0^{f}
		28	72.9 ± 2.8^{abcd}	82.9 ± 4.5^{ab}	70.9 ± 7.7^{abcd}	60.2 ± 11.5^{bcde}	36.1 ± 4.2^{de}	0^{f}
Great Salt Lake	24	21	85.4 ± 0.6^{a}	84.4 ± 2.3^{a}	87.2 ± 1.4^{a}	84.7 ± 0.2^{a}	17.5 ± 3.5^{c}	0^{d}
		28	86.1 ± 0.5^{a}	86.6 ± 1.7^{a}	88.6 ± 2.7^{a}	84.9 ± 2.6^{a}	27.2 ± 3.3^{b}	13.8 ± 6.7^{c}
	48	21	89.6 ± 1.2^{abcdef}	91.0 ± 1.0^{abcf}	89.8 ± 2.0^{abcdf}	89.5 ± 2.1^{abcdef}	24.9 ± 3.0^{i}	83.8 ± 3.1^{cdeg}
		28	89.0 ± 2.2^{abcdef}	87.5 ± 1.9^{abcdef}	84.6 ± 1.6^{bcdefg}	86.3 ± 1.9^{abcdefg}	34.6 ± 1.9^{h}	80.8 ± 1.7^{bcdeg}

Table 6. Survival (%) of Tibet A *Artemia* in medium of different salinity, ionic composition and temperature. For each culture period (days 4, 8, 11, 14 and 17) values with the same superscript are not significantly different (three-way ANOVA at $p = 0.05$); LC = Lagkor Co; DK = Dietrich & Kalle

Culture period		28 °C		21 °C	
		35 g.l^{-1}	80 g.l^{-1}	35 g.l^{-1}	80 g.l^{-1}
Day 4	LC water	66.3 ± 14.6^{abcd}	56.0 ± 20.1^{abcd}	77.2 ± 18.7^{abc}	69.0 ± 31.0^{abd}
	DK water	26.0 ± 7.0^{bcde}	3.0 ± 1.8^{cde}	50.5 ± 16.9^{abcde}	35.3 ± 9.8^{abcde}
Day 8	LC water	44.7 ± 15.3^{abde}	0^{cde}	62.2 ± 5.4^{abde}	48.7 ± 30.8^{abde}
	DK water	8.3 ± 3.9^{bcde}	0^{cde}	39.0 ± 17.4^{abcde}	19.8 ± 11.3^{bcde}
Day 11	LC water	16.7 ± 17.7^{bc}	0^{bc}	54.1 ± 6.9^{ab}	30.2 ± 22.2^{abc}
	DK water	5.8 ± 3.6^{bc}	0^{bc}	29.3 ± 15.3^{abc}	10.9 ± 9.0^{bc}
Day 14	LC water	0^{bc}	0^{bc}	42.9 ± 14.6^{ab}	22.7 ± 19.2^{abc}
	DK water	0^{bc}	0^{bc}	18.5 ± 12.6^{abc}	10.0 ± 0.85^{bc}
Day 17	LC water	0^{bc}	0^{bc}	39.0 ± 16.7^{ab}	16.3 ± 15.4^{ab}
	DK water	0^{bc}	0^{bc}	14.9 ± 9.7^{abc}	6.0 ± 0.42^{bc}

c) survival in medium of different salinity, ionic composition and temperature (Table 6)

There was a significant effect (three-way ANOVA, $p < 0.05$) of the ionic composition and temperature on survival of the Tibet A strain throughout the culture period. After 8 and 11 days of culture, there was also a significant effect of culture salinity on survival. Significant interactions between factors were only found for ionic composition vs. culture temperature at the end of the culture period (from 14 days onwards).

Generally, survival at the end of the test period was limited (39.0% was the highest final survival obtained). Survival was higher (though not always significantly) at 21 °C than at 28 °C: at the higher temperature, no live animals were observed after 2 weeks culture period in any medium, and in a thalassohaline medium of 80 g.l^{-1}, only 3% of the animals survived the first 4 days.

d) heat shock test (Fig. 2)

Viability of the cysts from Lagkor Co was minimal after the heat shock treatment, whereas the samples from Vietnam and San Francisco Bay proved most tolerant. The samples from Jingyu Lake and Co Qen took an intermediate position between those two extremes: about 50% of the cysts did not survive 15 min exposure to 50 °C, and a 60 min treatment resulted in complete inhibition of hatching for both strains.

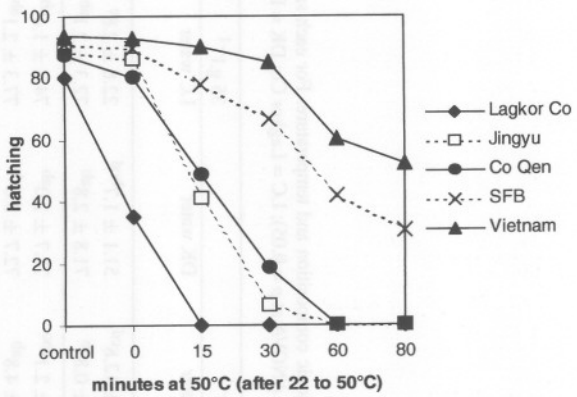

Figure 2. Hatching of cysts after heat shock of variable duration and subsequent incubation at 22.0 °C.

Species status of strains

a) sex ratio

The sex ratio (males/females) ranged between 45/55 and 40/60 for the Tibet A, Bozi Co, Bong Co and Jingyu Lake samples. For the samples from Haiyan Lake and Co Qen it was 32/68 and 11/89, respectively.

All samples had few ovoviviparously reproducing females. The status of the other females was unclear, as the large majority (>90%) had cysts in the uterus, but no spawning occurred. Only in Co Qen about 50% of the females with cysts in their uterus actually released cysts. The hatching percentage of this offspring after 2 weeks storage in brine at –18 °C was 23%.

Table 7. Cross breeding test of Jingyu (JY) strain with San Francisco Bay (SFB), Lagkor Co (LC) and Yuncheng (YC); n = 10; n.t. = not tested; H% = hatching percentage;% encyst = encystment rate; ovv. females = number of females (out of 10 replicates) producing partially or exclusively live nauplii. *F3 production by ovoviviparously generated F2 was not tested by lack of sufficient numbers of surviving F2 individuals

Cross (female × male)	F1 production (offspring/female/day)				
	cysts	nauplii	H% of cysts	% encyst	ovv. females
SFB × SFB	14.7 ± 8.8	8.6 ± 9.6	37.8	63.1	9
JY × JY	7.3 ± 6.8	1.8 ± 2.6	77.5	80.2	4
LC × LC	2.7 ± 2.8	2.1 ± 2.7	3.2	56.3	6
YC × YC	14.5 ± 8.5	9.8 ± 11.9	74.8	59.7	7
JY × SFB	2.7 ± 3.5	0	0	100	0
JY × LC	3.8 ± 4.0	0.5 ± 1.0	51.0	88.4	2
JY × YC	5.0 ± 4.1	3.2 ± 2.9	69.9	61.0	10
SFB × JY	14.6 ± 15.7	0	0	100	0
LC × JY	4.0 ± 3.6	1.0 ± 1.6	83.3	80.0	3
YC × JY	21.6 ± 11.3	1.9 ± 2.2	60.2	91.9	5

Cross (female × male)	F2 production (offspring/female/day) by oviparously generated F1					F2 production (offspring/female/day) by ovoviviparously generated F1				
	cysts	nauplii	H% of cysts	% encystm	ovv. females	cysts	nauplii	H% of cysts	% encystm	ovv. females
SFB × SFB	13.6 ± 7.1	3.4 ± 5.8	54.2	80.0	5	n.t.	n.t.	–	–	–
JY × JY	2.9 ± 2.5	1.1 ± 2.0	83.2	72.5	3	n.t.	n.t.	–	–	–
LC × LC	4.6 ± 3.2	1.6 ± 2.0	44.4	74.2	6	n.t.	n.t.	–	–	–
YC × YC	10.7 ± 4.2	8.0 ± 5.6	72.1	57.2	8	n.t.	n.t.	–	–	–
JY × SFB	–	–	–	–	–	–	–	–	–	–
JY × LC	7.5 ± 5.4	0.4 ± 1.0	63.5	94.9	2	4.4 ± 2.6	1.9 ± 1.1	63.4	69.8	5
JY × YC	9.6 ± 3.4	1.3 ± 2.0	71.1	88.1	5	10.0 ± 3.8	0.6 ± 1.1	71.2	94.3	3
SFB × JY	–	–	–	–	–	–	–	–	–	–
LC × JY	5.8 ± 3.0	1.0 ± 1.6	68.1	85.3	4	6.5 ± 3.2	1.0 ± 1.4	64.8	86.7	4
YC × JY	9.3 ± 4.7	2.3 ± 3.6	69.8	80.2	5	13.4 ± 5.3	1.2 ± 2.8	62.9	91.8	3

Cross (female × male)	F3 production* (offspring/female/day) by oviparously generated F2				
	cysts	nauplii	H% of cysts	% encystm	ovv. females
SFB × SFB	n.t.	n.t.	–	–	–
JY × JY	n.t.	n.t.	–	–	–
LC × LC	n.t.	n.t.	–	–	–
YC × YC	n.t.	n.t.	–	–	–
JY × SFB	–	–	–	–	–
JY × LC	4.1 ± 6.0	0.3 ± 1.3	59.3	93.2	1
JY × YC	4.9 ± 4.2	1.1 ± 2.2	65.2	81.7	2
SFB × JY	–	–	–	–	–
LC × JY	5.1 ± 6.0	1.7 ± 3.4	64.2	75.0	3
YC × JY	6.4 ± 5.9	1.1 ± 2.3	67.3	85.3	2

b) cross-breeding

Table 7 summarizes the results of the reciprocal crosses among Jingyu Lake (JY), Yuncheng (YC), Lagkor Co (LC) and SFB specimens, and shows the offspring (cysts or nauplii) per female per day for the successive generations, the hatching percentage of the

cysts produced, the encystment rate, and the number of ovoviviparously reproducing females in each combination.

Infertility of crosses between JY and SFB was illustrated by the production of non-hatching cysts and the absence of ovoviviparous offspring.

JY showed cross-fertility with both YC and LC, at least until F3. F3 cysts produced by these crosses showed hatching in the range 59.3–67.3% (51.0–83.3% in F1 generation) and a few F2 females (out of 10 replicates) reproduced ovoviviparously. The hatching percentage of the LC × LC cysts was low (3.2% in F1 cysts, 44.4% in F2 cysts), but the hibernation method was more effective in breaking diapause in the other combinations of crosses. The encystment rate did not show any clear trend over the different generations and combinations.

Discussion

Reports about the biotic elements of salt lakes at high altitude (>1000 m) are restricted. The aquatic fauna of the saltpans ('salares') on the South American Andes Altiplano (in Bolivia, extending into Peru and Chile) has been inventorized (Bayly, 1993; Dejoux, 1993; Williams et al., 1995), and together with other crustaceans, *Artemia* has been reported at altitudes of about 4000 m.

Much less has been published in international scientific literature about saline lakes in the vast (semi-) arid area of mountain ranges and high plateaux in the heart of the Asian continent (Russian Federation, China and Central Asian republics). A bisexual *Artemia* is reported in a number of Pamir salt lakes, among which Sasykkul in Tajikistan (Egorov, 1998), where the thermal regime is influenced by the presence of underground wells, which prevent the temperature of the bottom layer to drop below 0 °C in winter (Akhrorov, 2002).

Multidisciplinary study, using up-to-date *Artemia* characterisation techniques, identified *Artemia tibetiana* from Lagkor Co, Tibet (Abatzopoulos et al., 1998, 2002). No long-term ecological study of this lake (or any other on the Plateau) and its brine shrimp population is available, but the low resistance of this species to a standard heat shock test (as compared to strains from less extreme climates; Clegg et al., 2001) is explained as an adaptation to its environment. Another feature of the new species is the large size of its cysts (323.0–330.0 μm), larval (667 μm for instar I nauplii) and adult stage (Abatzopoulos et al., 1998).

The diameter of *A. tibetiana* cysts is nearly equalled by the sample from Jingyu Lake (320.0 μm). The Bozi Co population showed the smallest cyst size (284.5 μm), but this value is still in the range of the biggest parthenogenetic cysts reported, e.g. Margherita di Savoia (Italy) and Tuticorin (India), namely 280–285 μm, and well above values for current commercial samples like Great Salt Lake, 240–245 μm (Vanhaecke & Sorgeloos, 1980). Whatever the species status of the studied samples, the cysts are (very) big, especially compared to the other bisexual species from continental China, *A. sinica* from Yuncheng: 232 μm (Cai, 1989). This big cyst size has further been confirmed for new samples taken from four other Tibetan lakes, revealing a diameter in the range 291.0–358.1 μm (Yu et al., pers. comm.). Though a lot of other factors interfere (e.g. polyploid parthenogenetic strains are usually bigger sized than bisexuals) organisms in colder climates tend to be bigger than their counterparts at lower latitudes ('Bergmann's Rule'; Atkinson & Sibly, 1997). This was shown for freshwater copepods and cladocerans (Villalobos & Zuñiga, 1991; Gillooly & Dodson, 2000), rotifers (Stelzer, 2002), and perhaps is also illustrated by the size of the *Artemia* populations along a gradient of 20–50 ° south latitude on the South American Pacific coast (Gajardo et al., 1998).

The average instar I naupliar length (ranging between 540.2 and 607.1 μm, values for Haiyan and Jingyu Lake, respectively), though bigger than in most other strains (517 μm for Margherita di Savoia; Vanhaecke & Sorgeloos, 1980), is still far below the *A. tibetiana* value (667 μm; Abatzopoulos et al., 1998). No systematic research was done on the length of the adult animals.

Within the experimental conditions the cyst samples from the Qinghai-Tibet plateau showed higher hatching and survival at lower temperatures than the control strains from San Francisco Bay and Great Salt Lake. Despite this general pattern, the different strains didn't show an identical cold tolerance and/or preference, as is illustrated by the hatching pattern of Co Qen strain (Fig. 1) and the tolerance to heat shock of Co Qen and Jingyu Lake strains, as compared to Lagkor Co strain (Fig. 2). No *Artemia* is found in areas where year-round prevailing extremely low temperatures preclude its development (Persoone & Sorgeloos, 1980), but a lot of strains are found in the continental areas of North America

and Asia with extremely cold winter temperatures, but where high summer temperatures allow cyst hatching and subsequent colonization of the environment. *A. tibetiana* survives in a habitat with annual temperatures fluctuating between –26 and +24 °C, and with an average annual air temperature of ±1.6 °C (Zheng, 1997). For strains from less extreme climates hatching, growth and maturation are delayed below the range 25–30 °C, as shown by the control strains (GSL, SFB) in our tests; the exact temperature sensitivity however is strain-dependent (Reeve, 1963; Von Hentig, 1971; Vanhaecke et al., 1984; Thoeye et al. 1987; Browne et al., 1988; Vanhaecke & Sorgeloos, 1989).

Artemia species have been the subject of numerous salinity studies (Croghan, 1958a, b; Bowen et al., 1985; D'Agostino & Provasoli, 1986; Triantaphyllidis et al., 1995; Abatzopoulos et al., 2003), revealing population-specific physiological tolerances to salinities, specific ions and ionic ratios. *Artemia* can withstand environments in which the ratio of the major anions and cations may be totally different from that in seawater (Cole & Brown, 1967; Persoone & Sorgeloos, 1980; Bowen et al., 1988). Since the osmotic pressure differs in function of the salt composition, the costs of osmoregulation also differ in media of the same salinity but with various ionic environments. In our study the Lagkor Co and Tibet A populations performed better in the carbonate and sulfate enriched artificial Lagkor Co medium. The ionic composition of the habitat can result in ecological isolation of particular *Artemia* strains, as illustrated for *A. franciscana* (Bowen et al., 1985; 1988). In comparative tests (e.g. in our cross-breeding tests) the ionic composition of the common culture medium may therefore interfere with our results as the salt composition may not be optimal for all strains tested.

The high contents in HUFA's, and mainly EPA, is a recurring characteristic in Tibetan *Artemia*. EPA values in the range 19.2–46.6 $mg.g^{-1}$ dry weight have also been reported for cysts from four newly sampled lakes from this area (Yu et al., pers. comm.), which adds to the aquaculture potential of these strains, if size is not prohibitive. The HUFA profile of *Artemia* cysts reflects the feeding environment of the female parentals. Zheng (1997) reports *Dunaliella salina* and, to a lower extent, *Chlamydomonas* sp. as the main component of the phytoplankton flora in the saline lakes on the Qinghai-Tibet Plateau. *D. salina* has a high adaptation capacity to low temperature and to variable ionic composition, and is rich in proteins and β-carotene (Zheng, 1997). No data are given on the HUFA profile of these algae in the local conditions. In this respect, there may be a link between the HUFA pattern of the phytoplankton and the increased UV radiation at high altitudes. UV may affect virtually every aspect of life (survival, growth, reproduction, egg hatching, sex ratio) but effects may be very different between species and/or taxonomic groups (Häder et al., 1998; Sommaruga, 2001). The net effect on the food web may be extremely complex as all trophic levels are differently affected by UV, as shown in mesocosm and *in situ* enclosure experiments (Cabrera et al., 1997; Halac et al., 1997; Sommaruga et al., 1999). Generally bacterioplankton is affected to a greater extent than algae. The latter may thus be in a competitive advantage for nutrients, having an effect on their fatty acid contents (Plante & Arts, 2000). Other authors reported increased photodegradation of dissolved organic carbon as an effect of UV radiation, stimulating the food web (De Lange et al., 2003). Wangberg et al. (1999) found increased fatty acid content in marine phytoplankton at increased UV radiation levels.

The species status of the studied populations is not entirely clarified by our experiments. They all contained – to a variable degree – relatively high numbers of males and also parthenogenetically reproducing females. The sex ratio in the field, however, may differ from our laboratory data, due to possible selection during hatching and subsequent culture. Even in the field considerable seasonal fluctuations occur (Van Stappen et al., 2001). As these lakes have never been harvested systematically, the samples may be a mixture of cysts produced in different areas of the lake, spread over several seasons or years. The presence of parthenogenetic females in all samples complicates the determination of the species status of the bisexual individuals. Although this presence was minimal in the Jingyu Lake sample, our results do not allow confirmation of this bisexual sample as either *A. tibetiana* or *A. sinica*. Production of fertile offspring between individuals belonging to different 'Eastern Old World' *Artemia* bisexual species is not uncommon: Pilla & Beardmore (1994) successfully crossed *A. sinica* with *A. urmiana* and *Artemia* sp. from Kazakhstan, with no apparent hybrid breakdown at later generations (up to F3). Between *A. tibetiana* and *A. sinica*, partial fertility through F2 and F3 has been shown as well (Abatzopoulos et al., 2002). Cross-breeding through more successive generations might shed some more light on the species status of the Jingyu and other samples. Additionally, testing of alternative diapause deactivation

methods on cysts produced in cross-breeding tests may result in higher hatching: the low values obtained for LC × LC cysts (3.2% in F1, 44.4% in F2) suggests that the standard incubation of the cysts at –18 °C for two weeks was insufficient for optimal diapause breaking.

Though examples of natural coexistence of different Asian bisexual species (*A. urmiana*, *A. sinica* and *A. tibetiana*) are not known, there is evidence for coexistence of bisexual species with parthenogenetic populations, and for coexistence of different parthenogenetic strains, e.g. in Spain (Amat, 1980; 1983; Amat et al., 1995). Temporal cycling or niche partitioning may be the result of different relative fitness of the coexisting strains to the temperature profile of the environment (Browne, 1980; Browne et al., 1988; Browne & Halanych, 1989). Bowen et al. (1978) reported that parthenogenetic strains have more haemoglobin than sexual species, which might be advantageous at high salt concentration and at high altitudes. Partial coexistence has also been reported in the area of Lake Urmia, Iran, where there is evidence that the lake itself is the habitat of the bisexual species *A. urmiana* and a smaller fraction of parthenogenetic individuals, whereas in the adjacent lagoons and salt ponds with very different conditions of temperature and salinity only the parthenogenetic population is found (Van Stappen, 2002).

Techniques of DNA fingerprinting can result in a breakthrough in the understanding of genetic relationships between different populations and in the problem of coexistence of strains. In a database of 65 *Artemia* samples, based on RFLP patterns of a mitochondrial rDNA fragment (Wang et al., 2003), *A. tibetiana* from Lagkor Co clusters together with the samples from Tibet A and Bozi Co. The samples from Co Qen and Jingyu Lake, however, appear in this dendrogram in a large cluster of parthenogenetic and unidentified populations. All Tibetan samples show a large genetic distance from the *A. sinica* samples in this database. The high degree of diversity within each cluster, even among samples from the same habitat, illustrates that future analyses should focus on individuals, rather than on batches of cysts (Wang et al., 2003).

Conclusions

The *Artemia* biodiversity of PR China shows a complex pattern. The prevailing mode of reproduction in the coastal habitats in China is parthenogenesis (Xin et al., 1994), though in recent years some populations are mixed with, or have been outcompeted by, introduced *A. franciscana*. Numerous parthenogenetic populations also exist in inland lakes, but also bisexual populations are found in inland China, probably belonging to the species *A. sinica* (Van Stappen, 2002). The population of Lagkor Co, Tibet, has been identified as *A. tibetiana* (Abatzopoulos et al., 1998, 2002). Based on the available samples, our experiments show that bisexuals are also found in other lakes on the Qinghai-Tibet plateau, co-occurring with parthenogenetic populations. These populations share, to a variable degree, common characteristics like large cyst size, high HUFA content and tolerance to low temperatures. DNA fingerprinting techniques should bring decisive evidence on their exact species status and on the mixed nature of the populations.

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